possessed IL28B TT alleles and odds ratio calculation was impossible, IL28B SNPs was excluded from the analysis. Likewise, core aa.70, and IL28B were extracted as independent variables associated with nEVR (Table 4). In performing the analysis for SVR and relapse, we excluded the patients with extended length of therapy to standardize the treatment periods. Since this restriction reduced the number of available patients for the analysis, we included 30 additional patients (Supplementary Table 1) with available clinical information including HCV core, NS5A and IL28B SNPs. Those 30 patients were consecutively introduced the PEG-IFN/RBV therapy at Yamanashi University Hospital in succession to the initial 103 patients. As a result, 97 patients were available for SVR analysis, and 78 patients were available for relapse analysis. As shown in Table 5, ISDR aa.2224-2248, IRRDR aa.2340-2382, and IL28B SNPs were extracted as the independent variables affecting the SVR. On the other hand, IRRDR-V3 aa.2360-2377 was extracted as an independent factor for relapse (Supplementary Table 2).

Contribution of IL28B SNPs and NS5A aa.2340-2382 in Determining the Treatment Response

Since multivariate analysis finally extracted IL28B SNPs and IRRDR aa.2340-2382 as the two most significant variables determining the final outcome, the correlation of IL28B SNPs and IRRDR aa.2340-2382 in association with the final outcome was further investigated. In Fig.2, alignment of IRRDR aa.2340-2382 in association with SVR are demonstrated. By this analysis, it was evident that 3 or more mutations in IRRDR aa.2340-2382 were significantly associated with SVR. Lastly, to disclose the viral sequence contribution in determination of the final outcome in the IL28B TT haplotype patients with the standard therapy (n=47), sliding window analysis was performed (Fig. 1e). As demonstrated here, NS5A IRRDR aa.2340-2379(~2382) was finally extracted as the most significant viral region contributing the final outcome (p=2.47E-05).

In Fig.3, contribution of these three viral regions in the phase-specific treatment responses is schematically illustrated.

DISCUSSION

In this study, we determined 103 complete HCV ORF sequences in consecutive Japanese patients, infected with genotype-1b HCV and given PEG-IFN/RBV therapy, and systematically searched and investigated contribution of viral regions associated to the phase-specific treatment responses with IL28B SNP haplotypes. To our knowledge, this study is most comprehensive in these aspects: 1) complete HCV ORF studied with the largest analyzed number of patients, 2) analyzed according to viral kinetics closely related to the outcome, 3) unified to a single genotype, 1b, 4) unified background of patients, 5) introduction of a sliding window method to screen the responsible viral regions systematically, 6) analysis of IL28B SNPs.

In a recent randomized controlled study of PEG-IFN/RBV combination therapy, the status of the patients according to the response to the PEG-IFN/RBV therapy at 12 weeks showed a marked correlation with the final outcome, and the viral response at week 12 has been considered as a useful predictor in the early response-guided therapy (26). In agreement with the previous study, the virological responses to the PEG-IFN/RBV at week 12 had a distinct correlation with the final outcomes in our study group (SVR rate: 100%, 80%, 20%, and 0% for the RVR, cEVR, pEVR, and nEVR in the standard therapy). These results demonstrated that classification by the viral response at week 12 provides distinct groups with different characteristics.

We first tried to identify regions of the HCV ORF showing distinct linkage to the RVR and nEVR. We found that HCV substitutions around the ISDR (aa.2224-2248 in the RVR) were most significantly correlated with early viral clearance in PEG-IFN/RBV therapy. In contrast, core aa.70 substitution was most significantly correlated with nEVR demonstrating the association with treatment resistance. According to the results shown here, the early HCV dynamics in the PEG-IFN/RBV therapy are significantly regulated by the specific viral sequences in core and NS5A (Figs. 1a-b).

Next, we determined the HCV genomic region correlated with SVR of patients with standard therapy. We excluded the patients with extended therapy to unify the treatment duration. Considering the length of treatment, we first suspected that multiple factors might affect the final

outcome of 48 weeks' standard therapy and determining viral regions reflecting pure biological response would be difficult. Contrary to our prediction, a region almost identical to the IRRDR (aa.2340-2382) was extracted by systematic sliding analysis as correlated with the outcome, with significantly high p-value, demonstrating the remarkable influence of the IRRDR aa.2340-2382 in determining the final outcome (Fig. 1c). Importantly, in addition to the final outcome, when relapser and non-relapser in the ETR were compared, aa.2360-2377, the region almost coinciding with the V3 region of the IRRDR was extracted as the region discriminating these two groups (Fig. 1d).

In the analysis of IL28B SNPs (rs8099917), we observed significant correlation between IL28B SNP and viral dynamics at weeks 12; the patients with the minor/minor or minor/major alleles showed significantly poor responses, as demonstrated in Table 2. On the other hand, since poor response was significantly associated with the substitution of the core aa.70 as shown in Fig.1b in our study, we next tried to unveil the correlation between HCV ORF and IL28B SNPs. As demonstrated in Supplementary Fig.1, the significant link with the single core aa.70 substitution was observed through searching for the complete HCV ORFs. The result coincides with recent studies (27-29), and, moreover, confirms that this single spot is extraordinarily linked to the initial poor response amongst the complete 3010 HCV amino acid residues. Though the underlying mechanism for the association of IL28B and core aa.70 is unclear, the association would be reflection of an interaction between the IL28B SNPs and HCV sequences in the development of chronic HCV infection as discussed by Kurosaki et al (29). Namely, it is possible that HCV sequences within the patient might have been selected during the course of chronic infection depending on the IL28B SNPs by selective pressures of unknown mechanism.

By multivariate analysis, IL28B SNP, IRRDR aa.2340-2382, and ISDR aa.2224-2248 were extracted as independent variables related to the final outcome in patients with standard length of therapy with inclusion of additional 30 patients (Table 5). Among these, IL28B SNPs and IRRDR aa.2340-2382 were the two most significant variables determining the final outcome. Moreover, as demonstrated in Fig.1d, NS5A IRRDR aa.2340-2379(~2382) was the most significant viral region contributing the final outcome in patients with IL28B TT haplotype (p=2.47E-05), demonstrating

that combined information of the IL28B and IRRDR is significantly important in predicting viral kinetics and the treatment outcome.

Most of the viral genomic regions identified in this study have been already reported in previous, independent studies. However, the importance of our study is shown in the result that these specific viral regions of core, ISDR, and IRRDR were extracted all at once through systematic full HCV ORF sequence screening. What is unique in our study is the introduction of the sliding window analysis; through the analysis, we could effectively confine viral regions of ISDR and IRRDR that were not identified in other previous HCV ORF studies (21, 22). Furthermore, our study also disclosed that the importance of these viral regions was different according to each treatment-phase; RVR, nEVR, SVR and relapse were mostly related to the ISDR, core aa.70, the IRRDR and the IRRDR, respectively. The ISDR was first region identified as related to SVR in the era of IFN mono-therapy in Japanese patients, such that multiple mutations in the ISDR were associated with favorable IFN responses (10, 30). Contribution of the core region in the treatment response in IFN/RBV therapy was first reported by Akuta and colleagues, in that polymorphism of core aa.70 and 91 were closely related to the final outcome (20). The further significance of core polymorphism was reported in hepatocarcinogenesis as well (31, 32). Our analysis also confirmed the recent studies reporting the close correlation between the viral core and IL28B SNPs (11, 29, 32). The present finding that the core aa.70 is correlated with nEVR independently of IL28B seems to reflect the recent report that core aa.70 is an independent determinant of the poor response to the triple therapy of PEG-IFN/RBV and telaprevir in patients with IL28B minor allele (27). On the other hand, the IRRDR was originally reported by El-Shamy and colleagues as being related to the result of PEG-IFN/RBV therapy(15). Importantly, our study revealed that the final SVR and relapse were significantly correlated with mutations around the IRRDR. The result indicates its significant role in the late-phase viral responses in PEG-IFN/RBV therapy.

Core is a main component protein of viral nucleocapsid, and it is recently found that the core located on the surface of lipid droplets associates with NS5A to facilitate virion formation (33). HCV-JFH1 with core R70Q/H and L91M was reported to impair the virion formation resulting in

accumulation of intracellular core protein which causes the endoplasmic reticulum (ER) stress leading to IFN resistance through SOCS3 upregulation induced by IL-6 (34). NS5A is a phosphoprotein, and is considered to play a pivotal role both in viral replication and virion production depending on its phosphorylation state (35-37). Mutations in centrally located serine residues required for NS5A hyperphosphorylation as well as in its adjacently located ISDR work as adaptive mutations in HCV replicon possibly through decreasing hyperphosphorylated form of NS5A (37-40) which seems to control HCV replication. Conservation of c-terminal serine residual cluster of NS5A, downstream to IRRDR, is required for NS5A basal phosphorylation, interaction with the core protein on the lipid droplet, and thus virion formation (41, 42). Taken together, it can be speculated that the structural changes in core and NS5A protein can coordinately modify the HCV replication, especially through virion formation around lipid droplets. However, precise mechanism through which these modulations of the viral proteins lead to the different treatment response should be further investigated.

In conclusion, we have found that polymorphic viral sequences in core aa.70, NS5A-ISDR aa.2224-2248, and NS5A-IRRDR aa.2340-2382 in genotype-1b HCV infection are correlated significantly with the treatment phase-specific viral responses to PEG-IFN/RBV therapy. In addition, these viral responses were also significantly correlated with the polymorphism in IL28B SNP, and this polymorphism was significantly correlated with the polymorphism in the core. More importantly, combined information of the IL28B and IRRDR aa.2340-2382 is significantly important in predicting viral kinetics and the treatment outcome. We consider our comprehensive study provides a new basis for introducing the PEG-IFN/RBV therapy as well as new generation anti-HCV therapies.

FIGURE LEGENDS

Fig. 1

The contribution of viral sequences and IL28B SNPs (interleukin28B single nucleotide polymorphisms) in the treatment response to PEG-IFN (pegylated-interferon) plus RBV (ribavirin) was studied. Abbreviations: ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region; PKR-BD, PKR-binding domain.

(a) A sliding window analysis for RVR (rapid viral response) vs. the remainder is demonstrated (n=103).

(b) A single amino acid analysis for nEVR (non early viral response) vs. the remainder is demonstrated (n=103).

(c) A sliding window analysis for SVR (sustained viral response) vs. non-SVR is demonstrated (n=76).

(d) A sliding window analysis for relapsers vs. non-relapsers among ETR (end of treatment response) is demonstrated (n=57).

(e) A sliding window analysis for SVR vs. non-SVR in IL28B TT patients with the standard therapy (n=47).

Fig. 2 The alignment of NS5A region around IRRDR (IFN/RBV resistance-determining region) aa.2340-2382 is demonstrated along with SVR(sustained viral response).

Fig. 3 Roles of three HCV-1b viral regions in determination of the time-dependent treatment response to PEG-IFN/RBV therapy is illustrated. Abbreviations: IL28B SNPs, interleukin28B single nucleotide polymorphisms; RVR, rapid viral response; nEVR, non early viral response; SVR, sustained viral response.

Supplementary Fig. 1

The correlation between IL28B SNPs (interleukin28B single nucleotide polymorphisms) and HCV viral sequences were investigated by single amino acid comparison analysis (n=89). To do this, whole HCV sequences of TT patients (n=65) and that of TG/GG (n=24) patients were compared. Abbreviations: ISDR, interferon sensitivity-determining region; IRRDR, IFN/RBV resistance-determining region.

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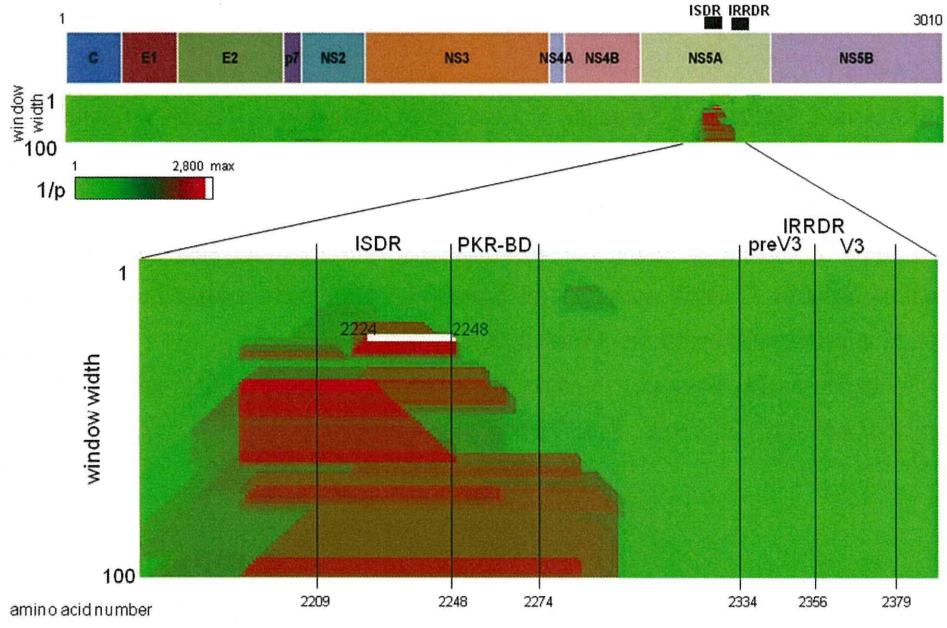
Maekawa et al Fig.1a



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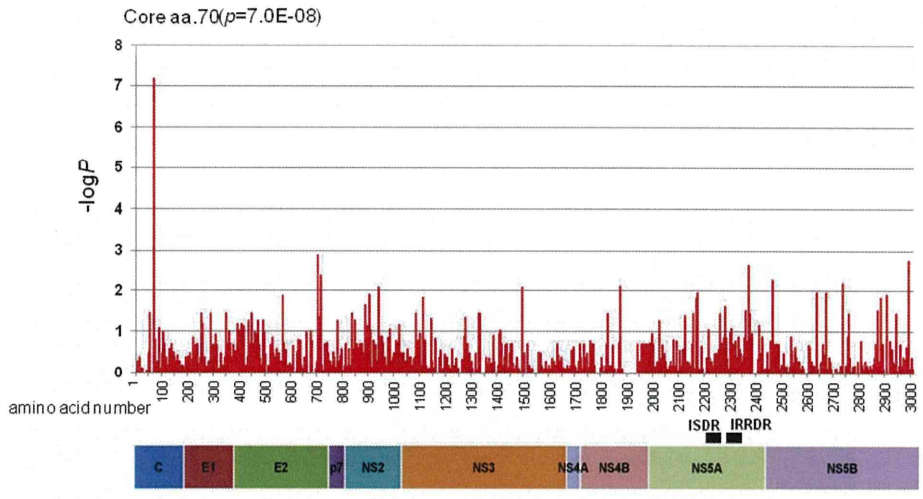
Maekawa et al. Fig.1b



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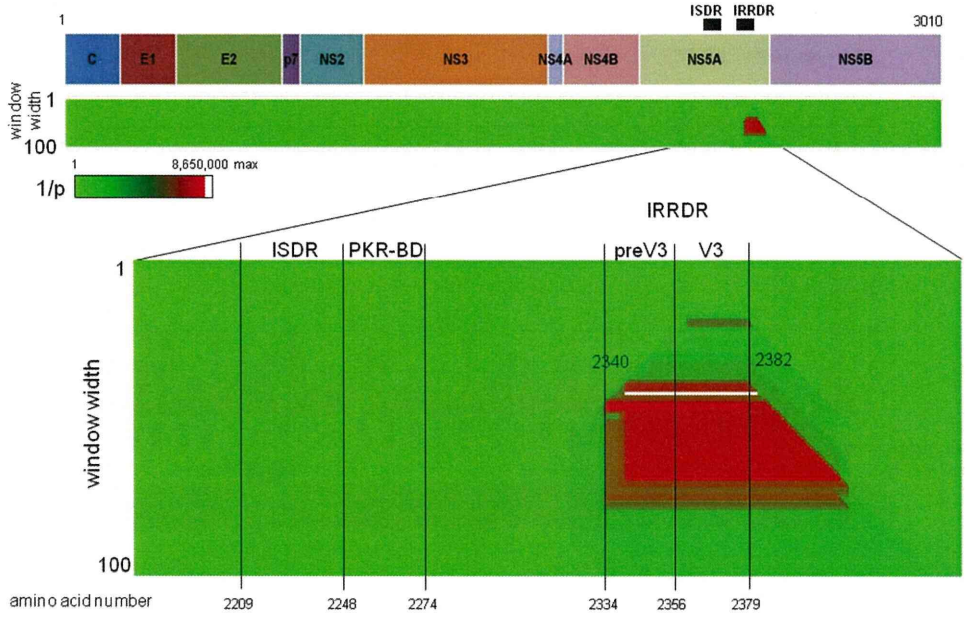

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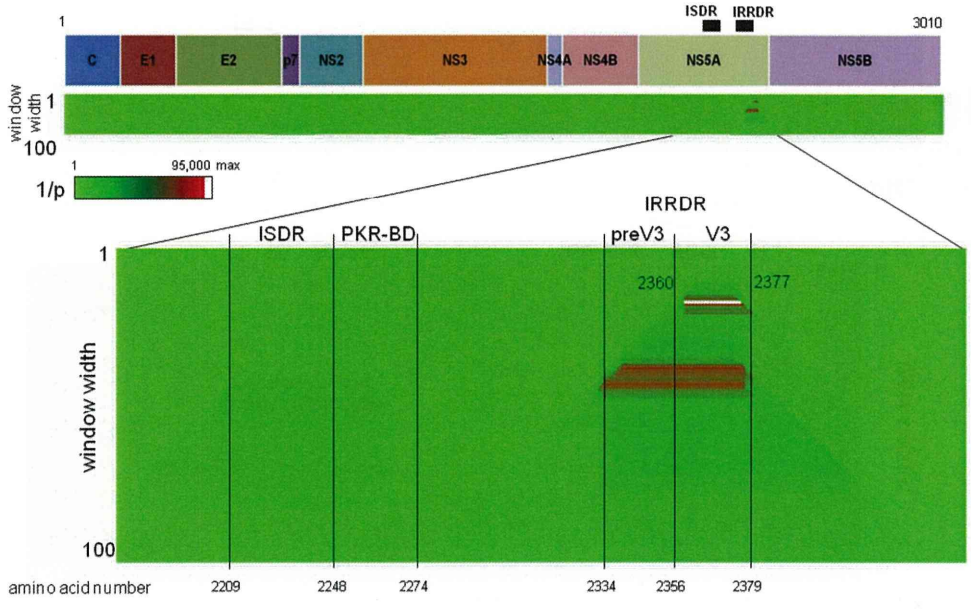

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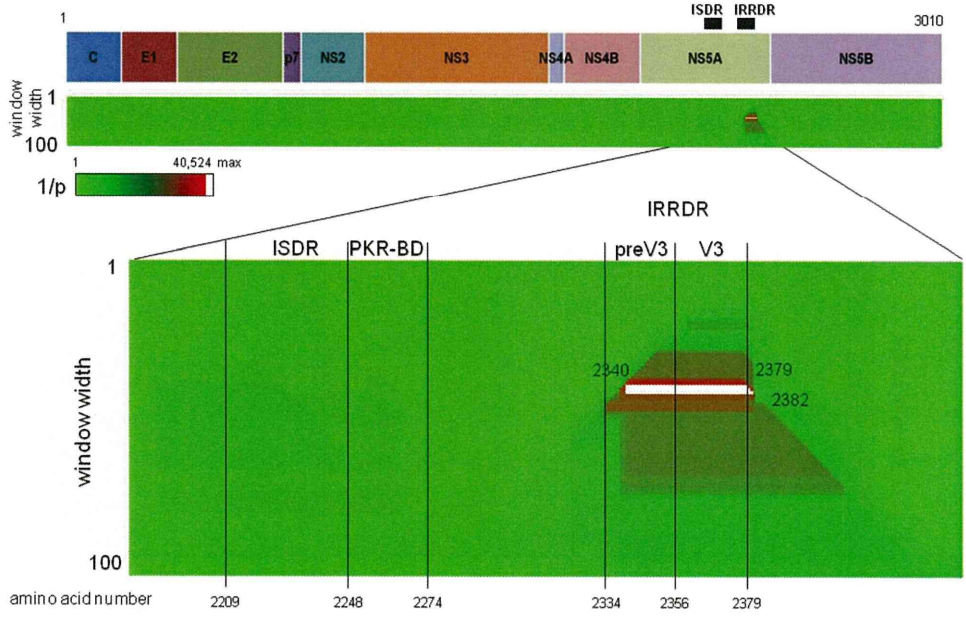

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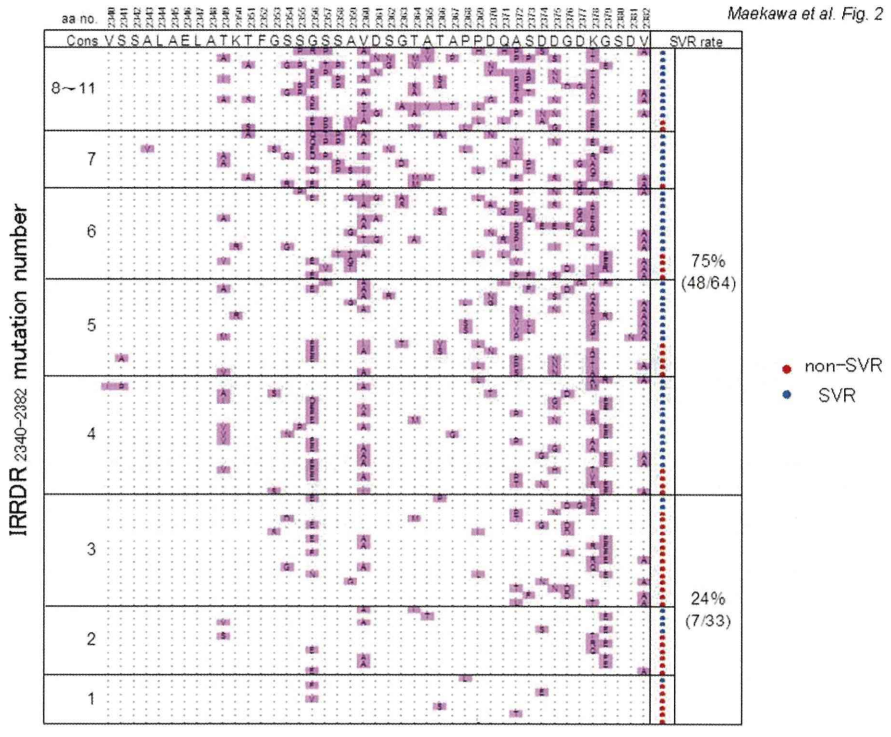

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Maekawa et al. Fig. 3

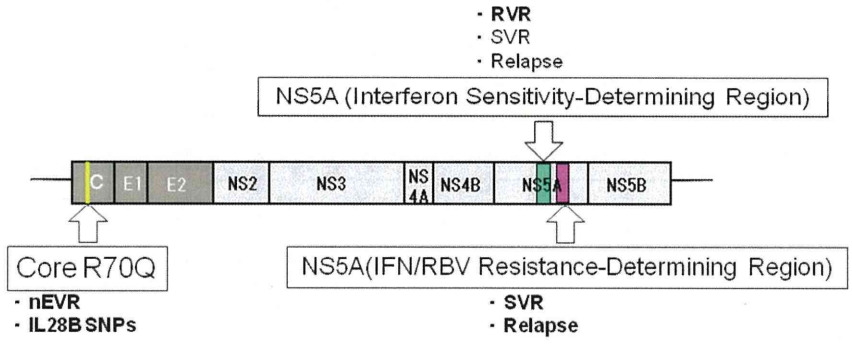

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Table 1. Baseline characteristics of 103 patients and SVR rate

Variables	Initial 103 patients
Age (years)	56 (31 - 70)
Gender :male	64 (62%)
Fibrosis : F2-F4	46 (44%)
HCV RNA (KIU/mL)	1500 (28 - 8392)
BMI	22.7 (17.5 - 31.7)
ALB (g/dL)	4.1 (3.0 - 4.9)
γGTP (IU/mL)	43 (11 - 289)
ALT (IU/mL)	68 (20 - 413)
T-Cho (mg/dL)	165 (104 - 240)
WBC (/μL)	4450 (2520 - 7850)
Hb (g/dL)	14.2 (11.2 - 17.9)
PLT (*10^4/μL)	14.5 (6.5 - 27.3)
AFP (ng/mL)	5.8 (0.7 - 468.4)
IL28B TT (%)	65 (73%) ¶
PEG-IFN dose (%)	89 (43 - 147)
Ribavirin dose (%)	98 (49 - 133)
SVR rate (n, %)	
All (n=103)	55 (53%)
Standard therapy (n=76)	
RVR (n=10)	10 (100%)
cEVR(n=35)	28 (80%)
pEVR(n=15)	3 (20%)
nEVR(n=16)	0 (0%)
Extended therapy (n=27)	
RVR (n=0)	0 -
cEVR(n=5)	3 (60%)
pEVR(n=18)	11 (61%)
nEVR(n=4)	0 (0%)

¶: n=89

Abbreviations: IL28B, interleukin 28B; SVR, sustained viral response; RVR, rapid viral response; cEVR, complete early viral response; pEVR, partial early viral response; nEVR, non early viral response.

Table 2. IL28B SNPs at rs8099917 and the initial viral responses (n=89)

	RVR (n=8)	cEVR-8w (n=17)	cEVR-12w (n=15)	pEVR (n=31)	nEVR (n=18)
TT	8(100%)	16(94%)	13(87%)	24(77%)	4(22%)
TG	0(0%)	1(6%)	1(7%)	7(23%)	12(67%)
GG	0(0%)	0(0%)	1(7%)	0(0%)	2(11%)

Abbreviations: IL28B SNPs, interleukin 28B single nucleotide polymorphisms; RVR, rapid viral response; cEVR, complete early viral response; pEVR, partial early viral response; nEVR, non early viral response.